(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 February 2003 (27.02.2003)

PCT

(10) International Publication Number
WO 03/016340 A1

(51) International Patent Classification: C07K 14/435

(21) International Application Number: PCT/EP02/09122

(22) International Filing Date: 15 August 2002 (15.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0120150.8 17 August 2001 (17.08.2001) GB

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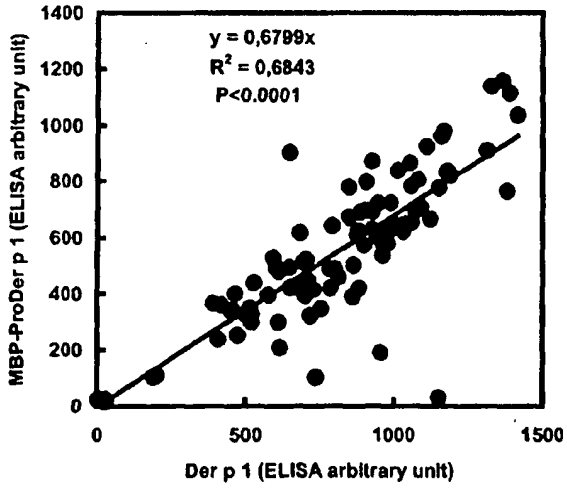
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK,

[Continued on next page]

(54) Title: DERP1 AND PRODERP1 ALLERGEN DERIVATIVES

Correlation between the IgE reactivity of MBP-ProDerP1 and natural DerP1.



(57) Abstract: The present invention provides a novel treatment for allergy comprising the provision of a recombinant DerP1/ProDerP1 allergen derivative with hypoallergenic activity. Pharmaceutical compositions comprising said mutant allergens which stimulate a Th1-type immune response in allergic or naïve individuals thereby reducing the potential for an allergic response upon contact with the wild-type allergen, are also provided.

WO 03/016340 A1

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TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DERP1 AND PRODERP1 ALLERGEN DERIVATIVES

The present invention relates to novel prophylactic and therapeutic formulations, said formulations being effective in the prevention and/or the reduction of allergic responses to specific allergens. Further this invention relates to hypoallergenic recombinant derivatives of the major protein allergen from *Dermatophagoides pteronyssinus,* allergen DerP1 and its precursor form ProDerP1. In particular the derivatives of the invention include physically modified DerP1 or ProDerP1 such as the thermally treated protein; or genetically modified recombinant DerP1 or ProDerP1 wherein one or more cystein residues involved in disulphide bond formation have been mutated. Methods are also described for expressing and purifying the DerP1 and ProDerP1 derivatives and for formulating immunogenic compositions and vaccines.

Allergic responses in humans are common, and may be triggered by a variety of allergens. Allergic individuals are sensitised to allergens, and are characterised by the presence of high levels of allergen specific IgE in the serum, and possess allergen specific T-cell populations which produce Th2-type cytokines (IL-4, IL-5, and IL-13). Binding of IgE, in the presence of allergen, to FcεRI receptors present on the surface of mastocytes and basophils, leads to the rapid degranulation of the cells and the subsequent release of histamine, and other preformed and neoformed mediators of the inflammatory reaction. In addition to this, the stimulation of the T-cell recall response results in the production of IL-4 and IL-13, together cooperating to switch B-cell responses further towards allergen specific IgE production. For details of the generation of early and late phase allergic responses see Joost Van Neeven *et al.*, 1996, Immunology Today, 17, 526. In non-allergic individuals, the immune response to the same antigens may additionally include Th1-type cytokines such as IFN-γ. These cytokines may prevent the onset of allergic responses by the inhibition of high levels of Th2-type immune responses, including high levels of allergen specific IgE. Importantly in this respect, is the fact that IgE synthesis may be controlled by an inhibitory feedback mechanism mediated by the binding of IgE/allergen complexes to the CD23 (FcεRII) receptor on B-cells (Luo et al., J.Immunol., 1991, 146(7), 2122-9; Yu et al., 1994, Nature, 369(6483):753-6). In systems that lack cellular bound CD23, this inhibition of IgE synthesis does not occur.

Type I allergic diseases mediated by IgE against allergens such as bronchial asthma, atopic dermatitis and perrenial rhinitis affect more than 20% of the world's population. Current strategies in the treatment of such allergic responses include means to prevent the symptomatic effects of histamine release by anti-histamine treatments and/or local administration of anti-inflammatory corticosteroids. Other strategies which are under development include those which use the hosts immune system to prevent the degranulation of the mast cells, Stanworth *et al.*, EP 0 477 231 B1. Other forms of immunotherapy have been described (Hoyne *et al.*, J.Exp.Med., 1993, 178, 1783-1788; Holt *et al.*, Lancet, 1994, 344, 456-458).

While immediate as well as late symptoms can be ameliorated by pharmalogical treatment, allergen-specific immunotherapy is the only curative approach to type I allergy. However, some problems related to this method remain to be solved. First, immunotherapy is currently performed with total allergen extracts which can be heterogeneous from batch to batch. Moreover, these allergen mixtures are not designed for an individual patient's profile and may contain unwanted toxic proteins. Second, the administration of native allergens at high doses can cause severe anaphylactic reactions and therefore the optimally efficient high dose of allergen for successful immunotherapy can often not be reached. The first problem has been addressed through alternative vaccination with better characterised and more reproducible recombinant allergens as compared to allergen extracts. The second problem, namely the risk of anaphylactic reactions induced by repeated injections of allergen extracts, can be minimised through the use of recombinant "hypoallergens", whose the IgE reactivity was altered by deletions or mutagenesis (Akdis, CA and Blaser, K, Regulation of specific immune responses by chemical and structural modifications of allergens, Int. Arch. Allergy Immunol., 2000, 121, 261-269).

Formulations have been described for the treatment and prophylaxis of allergy, which provide means to down-regulate the production of IgE, as well as modifying the cell mediated response to the allergen, through a shift from a Th2 type to a Th1 type of response (as measured by the reduction of ratio of IL-4 : IFN-γ producing DerP1 specific T-cells, or alternatively a reduction of the IL-5:IFN-γ ratio). This may for example be achieved through the use of recombinant allergens such as recDerP1 with reduced enzymatic activity as described in WO 99/25823. However the immunogenicity of these

recombinant allergens is thought to be similar to that of wild-type ProDerP1 in terms of IgE synthesis induction.

Non-anaphylactic forms of allergens with reduced IgE-binding activity have been reported. Allergen engineering has allowed a reduction of IgE-binding capacities of the allergen proteins by site-directed mutagenesis of amino acid residues or deletions of certain amino acid sequences. In the same time, T-cell activating capacity is still conserved as T cell epitopes are maintained. This has been shown using several approaches for different allergens although with variable results. Examples have been published for the timothy grass pollen allergen Phl p 5b (Schramm G et al., 1999, J Immunol.,162, 2406-14), for the major house dust mite allergens Derf2 (Takai et al. 2000, Eur. J. Biochem., 267, 6650-6656), DerP2 (Smith & Chapman 1996, Mol. Immunol. 33, 399-405) and Derf1 (Takahashi K et al. 2001, Int Arch Allergy Immunol.124, 454-60). One study has reported the generation of Derf1 hypoallergens by introductions of point mutations at the level of cysteine residues involved in disulfides bridges (Takahashi K Int Arch Allergy Immunol. 2001;124(4):454-60., Takai T, Yasuhara T, Yokota T, Okumura Y). However, if wild-type ProDerf1 was successfully secreted by *P. pastoris*, cysteine mutants concerning intramolecular disulfide bonds were, by contrast, not secreted.

The allergens from the house dust mite *Dermatophagoides pteronyssinus* are one of the major causative factors associated with allergic hypersensitivity reactions. Amongst these molecules, DerP1 is a an immunodominant allergen which elicits the strongest IgE-mediated immune response (Topham *et al.*, 1994, Protein Engineering, 7, 7, 869-894; Simpson *et al.*, 1989, Protein Sequences and Data Analyses, 2, 17-21) and with more than 75% of allergic patients to dust mites who develop IgE directed to this allergen. Hypoallergen derived from house dust mite DerP1, and effective prophylactic as well as therapeutic vaccine against this allergen have never been described.

The present invention relates to the provision and use of recombinant derivatives of *Dermatophagoides pteronyssinus* DerP1 allergen or of its precursor form ProDerP1 thereafter referred to as "DerP1/ProDerP1", with reduced allergenic activity compared to the wild-type allergen. The recombinant forms of DerP1 derivatives according to the invention, either adjuvanted recombinant proteins or plasmid encoding DerP1/ProDerP1 suitable for NAVAC, are used as prophylactic or therapeutic vaccines to induce strong

preventive Th1 or to shift Th2 to Th1 immune responses. The hypoallergenic derivatives can be successfully produced in recombinant expression systems and this is also an aspect of the present invention.

DerP1 is a 30 KDa protein and has been cloned and sequenced (Chua *et al.*, 1988, J.Exp.Med., 167, 175-182). It is known to contain 222 amino acid residues in the mature protein. The sequence of DerP1 shares 31% homology to papain, and shares more particularly homology in the enzymatically active regions, most notably the Cys34-His170 ion pair (*Topham et al., supra*). DerP1 is produced in the mid-gut of the mite, where its role is probably related to the digestion of food. Up to 0.2 ng or proteolytically active DerP1 is incorporated into each fecal pellet, each around 10-40 μm in diameter and, therefore, easily inspired into the human respiratory tract. Overnight storage of purified DerP1 preparations at room temperature results in almost complete loss of enzymatic activity due to autoproteolytic degradation (Machado *et al.*, 1996, Eur.J.Immunol. 26, 2972-2980). The DerP1 encoding cDNA sequence reveals that, like many mammalian and plant proteinases, DerP1 is synthetised as an inactive preproenzyme of 320 amino acid residues which is subsequently processed into a 222-amino acid mature form (Chua *et al.*, 1988, J.Exp.Med., 167, 175-182; Chua et al., 1993, Int. Arch Allergy Immunol 101, 364-368). The maturation of ProDerP1 is not known to date but it is thought that the allergen is processed by the cleavage of the 80-residues proregion.

The present invention provides a recombinant *Dermatophagoides pteronyssinus* DerP1/ProDerP1 protein allergen derivative wherein said allergen derivative has a significantly reduced allergenic activity compared to that the wild-type allergen. The allergenic activity can be impaired by several means which all aim at destructuring the protein forms by disrupting its intramolecular disulphide bridges thereby destabilising its 3-dimensional structure. Said allergen derivatives having the following advantages over the unaltered wild-type allergen: 1) increases the Th1-type aspect of the immune responses (higher IgG2a for example) in comparison to those stimulated by the wild type allergen, thereby leading to the suppression of allergic potential of the vaccinated host, 2) having reduced allergenicity while still retaining T cell reactivity, thus being more suitable for systemic administration of high doses of the immunogen, 3) will induce DerP1 specific IgG which compete with IgE for the binding of native DerP1, 4)

efficiently protects against airway eosinophilia even after exposure to aerosolised allergen extract. Such derivatives are suitable for use in therapeutic and prophylactic vaccine formulations which are suitable for use in medecine and more particularly for the treatment or prevention of allergic reactions.

According to a first aspect, the present invention provides a recombinant DerP1/ProDerP1 (i.e. DerP1 or ProDerP1) allergen derivative wherein the allergenic activity has been significantly reduced, e.g. almost or completely abolished, by a physical means such as by thermally treating the protein, preferably in the presence of a reducing agent. Typically, the DerP1/ProDerP1 protein is treated during a few minutes at about 100°C in the presence of a reducing agent. Preferably the reducing agent is beta-mercaptoethanol or DTT. Still more preferably the protein is treated during 5 minutes at about 100°C in the presence of 50 mM beta-mercaptoethanol. This treatment has a detrimental effect on the stability of the protein conformational IgE-binding epitopes.

In a second aspect the present invention provides a recombinant DerP1/ProDerP1 protein derivative wherein the allergenic activity has been genetically impaired such as by introducing specific mutations into the encoding cDNA or the genomic DNA. Accordingly an aspect of the invention provides the genetically mutated recombinant DerP1/ProDerP1 *per se.* The reduction of the allergenicity of DerP1/ProDerP1 may be performed by introducing mutations into the native sequence before recombinantly producing the hypoallergenic mutants. This may be achieved by: introducing substitutions, deletions, or additions in or by altering the three dimensional structure of the protein such that the tridimensional conformation of the protein is lost. This may be achieved, amongst others, by expressing the protein in fragments, or by deleting cysteine residues involved in disulphide bridge formation, or by deleting or adding residues such that the tertiary structure of the protein is substantially altered. Preferably, mutations may be generated with the effect of altering the interaction between two cysteine residues, typically one mutation at positions 4, 31, 65, 71, 103 and 117 of the native – mature - DerP1 (which corresponds to positions 84, 111, 145, 151, 183 and 197 of ProDerP1, respectively). A mutated protein according to the invention may comprise two or more (3, 4, 5 or all 6) cysteine mutations, thereby affecting different disulphide bridges, such as mutations at positions 4 & 31, 4 & 65, 4 & 71, 4 & 103, 31 & 65, or 4 & 31 & 65, or at positions 71 & 103, 71 & 117, 103 & 117, 31 & 117, 65 & 117, or 71 & 103 & 117.

Preferably the derivatives comprise one single mutation at any of the above positions. The most preferred mutation involves Cys4 (or alternatively, or in addition, Cys117 which is thought to be the disulphide bond partner of Cys4). The Cys mutations can be deletions, but are preferably substitutions for any of the other natural 19 amino acids. Preferred substitutions introduce positively charged amino acid residues to further destabilise the 3D-structure of the resulting protein. For example, preferred substitutions involve cysteine→arginine (or lysine) substitution.

Accordingly, the invention is illustrated herein by, but is not limited to, six specific mutations which are given as examples of hypoallergenic DerP1/ProDerP1 derivatives. First the allergenic activity of ProDerP1 is substantially reduced, preferably completely abrogated by substituting a cysteine residue for an arginine residue at position Cys4 of DerP1 protein sequence, and is set out in SEQ ID NO:3. Second, the allergenic activity of ProDerP1 is substantially abrogated by substituting a cysteine residue for an arginine residue at any of the following positions (calculated by reference to the sequence in mature DerP1): Cys31 of DerP1 protein sequence (SEQ ID NO:5), Cys65 (SEQ ID NO:7), Cys71 (SEQ ID NO:9), Cys103 (SEQ ID NO:11), Cys117 (SEQ ID NO:13).

Mutated versions of DerP1/ProDerP1 may be prepared by site-directed mutagenesis of the cDNA which codes for the DerP1/ProDerP1 protein by conventional methods such as those described by G. Winter *et al* in Nature 1982, 299, 756-758 or by Zoller and Smith 1982; Nucl. Acids Res., 10, 6487-6500, or deletion mutagenesis such as described by Chan and Smith in Nucl. Acids Res., 1984, 12, 2407-2419 or by G. Winter et al in Biochem. Soc. Trans., 1984, 12, 224-225.

The invention is not limited to the specifically disclosed sequence, but includes any hypoallergenic allergen which has been mutated to decrease or abolish its IgE-binding reactivity and/or histamine release activity, whilst retaining its T cell reactivity and/or the ability to stimulate an immune response against the wild-type allergen. The allergenic activity, and consequently the reduction in the allergenic activity, of the mutant allergens may be compared to the wild type by any of the following methods: histamine release activity or by IgE-binding reactivity, according to the method detailed in the Example section.

"Substantially reduced allergenic activity" means that the allergenic activity as measured by residual IgE-binding activity is reduced to a maximum of 50% of the

activity of the native – unmodified or unmutated - protein, preferably to a maximum of 20%, more preferably to a maximum of 10%, still more preferably to a maximum of 5%, still more preferably to less than 5%. Alternatively, "substantially" also means that the histamine release activity of the mutant is reduced by at least a 100-fold factor as compared to the native protein, preferably by a factor of 1000-fold, still more preferably by a factor of 10000-fold.

The immunogenicity of the mutant allergen may be compared to that of the wild-type allergen by various immunologicals assays. The cross-reactivity of the mutant and wild-type allergens may be assayed by *in vitro* T-cell assays after vaccination with either mutant or wild-type allergens. Briefly, splenic T-cells isolated from vaccinated animals may be restimulated *in vitro* with either mutant or wild-type allergen followed by measurement of cytokine production with commercially available ELISA assays, or proliferation of allergen specific T cells may be assayed over time by incorporation of tritiated thymidine. Also the immunogenicity may be determined by ELISA assay, the details of which may be easily determined by the man skilled in the art. Briefly, two types of ELISA assay are envisaged. First, to assess the recognition of the mutant DerP1 by sera of mice immunized with the wild type DerP1; and secondly by recognition of wild type DerP1 allergen by the sera of animals immunised with the mutant allergen. Briefly, each wells will be coated with 100 ng of purified wild type or mutated DerP1 overnight at 4°C. After incubating with a blocking solution (TBS-Tween 0.1% with 1% BSA) successive dilutions of sera will be incubated at 37°C for 1 hour. The wells are washed 5 times, and total IgG revealed by incubating with an anti-IgG antibody conjugated with Alkaline phosphatase.

A further aspect of the present invention provides an isolated nucleic acid encoding a mutated version of the DerP1/ProDerP1 allergen as disclosed herein. Preferably the nucleotide sequence is a DNA sequence and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al* in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by a combination of these techniques. Preferably the nucleic acid sequence has a codon usage pattern that has been optimised so as to mimic the one used in the intended expression host, more preferably resembling that of highly expressed mammalian e.g. human genes. Preferred DNA sequences are codon-optimised sequences

and are set out in SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12 and SEQ ID NO:14.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50ml or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801.

Alternatively, the coding sequence can be derived from DerP1/ProDerP1 mRNA, using known techniques (e.g. reverse transcription of mRNA to generate a complementary cDNA strand), and commercially available cDNA kits.

Desirably the codon usage pattern of the nucleotide sequence is typical of highly expressed human genes. Accordingly there is provided in a particular aspect of the invention a nucleotide sequence comprising a plurality of codons together encoding the mutated DerP1/ProDerP1 protein, wherein the selection of the possible codons used for encoding the recombinant mite protein amino acid sequence has been changed to closely mimic the optimised mammalian codon usage, such that the frequency of codon usage in the resulting gene sequence is substantially the same as a mammalian gene which would

encode the same protein. Codon usage patterns for mammals, including humans, can be found in the literature (see e.g. Nakamura et al. 1996, Nucleic Acids Res. 24, 214-215).

The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilization of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria, insect and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in *E.coli* or a viral gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. However, a gene with a codon usage pattern suitable for *E.coli* expression may also be efficiently expressed in humans. It is believed that the presence in a heterologous DNA sequence of clusters of codons which are rarely observed in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

There are several examples where changing codons from those which are rare in the host to those which are host-preferred ("codon optimisation") has enhanced heterologous expression levels, for example the BPV (bovine papilloma virus) late genes L1 and L2 have been codon optimised for mammalian codon usage patterns and this has been shown to give increased expression levels over the wild-type HPV sequences in mammalian (Cos-1) cell culture (Zhou et. al. J. Virol 1999. 73, 4972-4982). In this work, every BPV codon which occurred more than twice as frequently in BPV than in mammals (ratio of usage >2), and most codons with a usage ratio of >1.5 were conservatively replaced by the preferentially used mammalian codon. In WO97/31115, WO97/48370 and

WO98/34640 (Merck & Co., Inc.) codon optimisation of HIV genes or segments thereof has been shown to result in increased protein expression and improved immunogenicity when the codon optimised sequences are used as DNA vaccines in the host mammal for which the optimisation was tailored.

In this work, the sequences preferably consist entirely of optimised codons (except where this would introduce an undesired restriction site, intron splice site etc.) because each *D. pteronyssinus* codon is conservatively replaced with the optimal codon for a mammalian host. Surprisingly such optimised ProDerP1/DerP1 sequences also express very well in yeast despite the different codon usage of yeast.

A still further aspect of the invention provides a process for the preparation of a mutated DerP1/ProDerP1 protein which process comprises expressing DNA, either codon optimised or not, encoding the said protein in a recombinant host cell and recovering the product.

Although DerP1 is well characterized in terms of its enzymatic activity, allergenicity and gene cloning, heterologous expression of DerP1 has been reported to be problematic (Chapman and Platts-Mills, J Immunol 1980;125:587-592), probably because this cysteine proteinase is synthesized as a PreProDerP1 precursor. Even more problematic is the expression of DerP1/ProDerP1 sequences wherein cysteine residues involved in the protein conformation have been mutated. Accordingly the present invention further provides a process overcoming all these drawbacks therefore allowing the production of the mutated proteins and the industrial development of therapeutic and prophylactic vaccines to mite allergy.

A substantial amelioration of protein expression has been achieved in *E. coli* when DerP1/ProDerP1 either mutated or not was expressed as a Maltose Binding Protein (MBP) fusion protein. Accordingly there is provided a process for expressing the mutated ProDerP/DerP1 protein as a MBP fusion protein in *E. coli*. Furthermore, a substantial amelioration of protein expression in yeast has been surprisingly achieved for the mutated protein even though disulphide bonds are said to be essential for secretion in *Pichia pastoris* (Takai et al. 2001, Int. Arch. Allergy Immunol. 124, 454-460). This was achieved by re-engineering the polynucleotide sequence which encodes the *Dermaphagoides* mutated ProDerP/DerP1 protein to fit the codon usage found in highly expressed human genes, thereby also allowing the recombinant antigen to have the same

conformation and immunological properties as native ProDerP/DerP1 *Dermaphagoides* allergens. Surprisingly, the cloning and expression of mutated ProDerP1, codon-optimised for mammalian cell expression, could be achieved in *Pichia pastoris,* with a certain proportion being secreted, although expression in *P. pastoris* has been formerly reported to be unsuccessful (Takai et al. 2001, Int. Arch. Allergy Immunol. 124, 454-460).

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et. al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of:

1. Preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the said DerP1/ProDerP1 protein;
2. Altering the IgE-binding activity of the resultant protein by replacing the cysteine residues involved in disuphide bonds with another residue, preferably an arginine residue, using site directed mutagenesis;
3. Transforming a host cell with the said vector
4. Culturing the transformed host cell under conditions permitting expression of the DNA polymer to produce the protein; and
5. Recovering the protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vector is novel and also forms part of the invention. One particular aspect of the present invention provides an expression vector which comprises, and is capable of directing the expression of, a polynucleotide sequence encoding a cystein-mutated DerP1/ProDerP1 protein according to the invention. Another particular aspect of

the invention provides an expression vector which comprises, and is capable of directing the expression of, a polynucleotide sequence encoding a cysteine-mutated DerP1/ProDerP1 protein wherein the codon usage pattern of the polynucleotide sequence is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector may be suitable for driving expression of heterologous DNA in bacterial, insect, yeast or mammalian cells, particularly human cells.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the DerP1/ProDerP1 protein under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of $CaCl_2$ (Cohen *et al*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells, by lipofection, or by electroporation. Yeast compatible vectors also carry markers that allow the selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy

metals on wild-type strains. Control sequences for yeast vectors include promoters for glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 1968, 7, 149), PHO5 gene encoding acid phosphatase, CUP1 gene, ARG3 gene, GAL genes promoters and synthetic promoter sequences. Other control elements useful in yeast expression are terminators and leader sequences. The leader sequence is particularly useful since it typically encodes a signal peptide comprised of hydrophobic amino acids, which direct the secretion of the protein from the cell. Suitable signal sequences can be encoded by genes for secreted yeast proteins such as the yeast invertase gene and the a-factor gene, acid phosphatase, killer toxin, the a-mating factor gene and recently the heterologous inulinase signal sequence derived from INU1A gene of Kluyveromyces marxianus. Suitable vectors have been developed for expression in *Pichia pastoris* and *Saccharomyces cerevisiae.*

A variety of *P. pastoris* expression vectors are available based on various inducible or constitutive promoters (Cereghino and Cregg, FEMS Microbiol. Rev. 2000,24:45-66). For the production of cytosolic and secreted proteins,the most commonly used P. pastoris vectors contain the very strong and tightly regulated alcohol oxidase (AOX1) promoter. The vectors also contain the *P. pastoris* histidinol dehydrogenase (HIS4) gene for selection in his4 hosts. Secretion of foreign protein require the presence of a signal sequence and the *S. cerevisiae* prepro alpha mating factor leader sequence has been widly and successfully used in Pichia expression system. Expression vectors are integrated into the P. pastoris genome to maximize the stability of expression strains. As in *S.cerevisiae*, cleavage of a *P. pastoris* expression vector within a sequence shared by the host genome (AOX1 or HIS4) stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus. In general, a recombinant strain that contains multiple integrated copies of an expression cassette can yield more heterologous protein than single-copy strain. The most effective way to obtain high copy number transformants requires the transformation of *Pichia* recipient strain by the sphaeroplast technique (Cregg et all 1985, Mol.Cell.Biol. 5: 3376-3385).

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al*

and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as *E. coli* it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, absorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

Alternatively, the expression may be carried out either in insect cells using a suitable vector such as a baculovirus, in transformed drosophila cells, or mammalian CHO cells. The novel protein of the invention may also be expressed in yeast cells as described for the CS protein in EP-A-0 278 941.

Pharmaceutical, immunogenic and vaccine compositions comprising a hypoallergenic DerP1/ProDerP1 derivative according to the invention, or the polynucleotide sequences encoding said proteins, either codon-optimised or not, are also provided. In preferred embodiments the DNA composition comprises a plurality of particles, preferably gold particles, coated with DNA comprising a vector encoding a polynucleotide sequence which encodes a *D. pteronyssinus* amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence is typical of highly expressed mammalian genes, particularly human genes.

The polynucleotides and encoded polypeptides according to the invention may find use as therapeutic or prophylactic agents. In particlular the polynucleotides of the invention (including a polynucleotide sequence of native ProDerP1 – preferably codon optimised) may be used in DNA vaccination (NAVAC), the DNA being administered to the mammal e.g. human to be vaccinated. The nucleic acid, such as RNA or DNA, preferably DNA, is provided in the form of a vector, such as those described above, which may be expressed in the cells of the mammal. The polynucleotides may be administered by any available technique. For example, the nucleic acid may be introduced by needle injection, preferably intradermally, subcutaneously or intramuscularly. Alternatively, the nucleic acid may be delivered directly into the skin using a nucleic acid delivery device such as particle-mediated DNA delivery (PMDD). In

this method, inert particles (such as gold beads) are coated with a nucleic acid, and are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin), for example by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are delivery devices loaded with such particles).

Suitable techniques for introducing the naked polynucleotide or vector into a patient include topical application with an appropriate vehicle. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). DNA uptake may be further facilitated by use of facilitating agents such as bupivacaine, either separately or included in the DNA formulation. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901. Typically the nucleic acid is administered in an amount in the range of 1pg to 1mg, preferably 1pg to 10μg nucleic acid for particle mediated gene delivery and 10μg to 1mg for other routes.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral vector systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral, Canarypox and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids, microsphere encapsulation technology (poly(lactide-co-glycolide) and, liposome-based systems. Viral and non-viral delivery systems may be combined where it is desirable to provide booster injections after an initial vaccination, for example an initial "prime" DNA vaccination using a non-viral vector such as a plasmid followed by one or more "boost" vaccinations using a viral vector or non-viral based system.

In this way, the inventors have found that vaccination with DNA encoding ProDerP1 (preferably codon optimised for mammals) induces a Th1 response in mice models (high titres of specific IgG2a antibodies and low totres of specific IgG1) and, remarkably, the absence of anti-ProDerP1 IgE.

The pharmaceutical compositions of the present invention may include adjuvant compounds, or other substances which may serve to increase the immune response induced by the protein.

The vaccine composition of the invention comprises an immunoprotective amount of the mutated version of the DerP1/ProDerP1 hypoallergenic protein. The term "immunoprotective" refers to the amount necessary to elicit an immune response against a subsequent challenge such that allergic disease is averted or mitigated. In the vaccine of the invention, an aqueous solution of the protein can be used directly. Alternatively, the protein, with or without prior lyophilization, can be mixed, adsorbed, or covalently linked with any of the various known adjuvants.

Suitable adjuvants are commercially available such as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, and chemokines may also be used as adjuvants.

In the formulations of the invention it is preferred that the adjuvant composition induces an immune response predominantly of the TH1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favour the induction of cell mediated immune responses to an administered antigen. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Accordingly, suitable adjuvants for use in eliciting a predominantly Th1-type response include, for example a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. Other known adjuvants, which preferentially induce a TH1 type immune response, include CpG containing oligonucleotides. The oligonucleotides are characterised in that the CpG

dinucleotide is unmethylated. Such oligonucleotides are well known and are described in, for example WO 96/02555. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science 273*:352, 1996. CpG-containing oligonucleotides may also be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 as disclosed in WO 00/09159 and WO 00/62800. Preferably the formulation additionally comprises an oil in water emulsion and/or tocopherol.

Another preferred adjuvant is a saponin, preferably QS21 (Aquila Biopharmaceuticals Inc., Framingham, MA), that may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), Detox (Ribi, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs).

Accordingly there is provided an immunogenic composition comprising a DerP1/ProDerP1 hypoallergenic derivative as disclosed herein and an adjuvant, wherein the adjuvant comprises one or more of 3D-MPL, QS21, a CpG oligonucleotide, a polyethylene ether or ester or a combination of two or more of these adjuvants. The DerP1/ProDerP1 hypoallergenic derivative within the immunogenic composition is preferably presented in an oil in water or a water in oil emulsion vehicle.

In a further aspect, the present invention provides a method of making a pharmaceutical composition including the step of mutating one or more cysteine residues involved in disulphide bridge formation, such as Cys4, Cys31, Cys65, Cys71, Cys103 or

Cys117. The method further comprises the step of altering the codon usage pattern of a wild-type DerP1/ProDerP1 nucleotide sequence, or creating a polynucleotide sequence synthetically, to produce a sequence having a codon usage pattern typical of highly expressed mammalian genes and encoding a codon-optimised cysteine-mutated ProDerP1/DerP1 amino acid sequence according to the invention. Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds. Powell M.F. & Newman M.J). (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, US Patent 4,372,945 and Armor *et al.*, US Patent 4,474,757.

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-200 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. The vaccines of the present invention may be administered to adults or infants, however, it is preferable to vaccinate individuals soon after birth before the establishment of substantial Th2-type memory responses. Following an initial vaccination, subjects will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of allergic responses exists.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The present invention also provides a process for the production of a vaccine, comprising the steps of purifying a DerP1/ProDerP1 derivative according to the invention

or a derivative thereof, by the process disclosed herein and admixing the resulting protein with a suitable adjuvant, diluent or other pharmaceutically acceptable excipient.

The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient.

Another aspect of the invention is the use of a protein or polynucleotide as claimed herein for the manufacture of a vaccine for immunotherapeutically treating a patient susceptible to or suffering from allergy. A method of treating patients susceptible to or suffering from allergy comprising administering to said patients a pharmaceutically active amount of the immunogenic composition disclosed herein is also contemplated by the present invention.

A further aspect of the invention provides a method of preventing or mitigating an allergic disease in man (particularly house dust mite allergy), which method comprises administering to a subject in need thereof an immunogenically effective amount of a mutated allergen of the invention, or of a vaccine in accordance with the invention.

FIGURE LEGENDS

Figure 1: IgG and IgE-binding reactivity of denatured ProDerP1 expressed in CHO cells. Immunoplates were coated with 500ng/well of purified native or denatured ProDerP1 and incubated with sera (diluted 1:8) radioallergosorbent positive to *D. pteronyssinus*. Bound IgE or IgG were quantitated by incubation with mouse anti-human IgE or IgG and alkaline phosphatase-labelled anti-mouse IgG antibodies, followed by an enzymatic assay. Results are expressed as OD_{410nm} values.

Figure 2: Correlation between the IgE reactivity of MBP-ProDerP1 and natural DerP. Immunoplates were coated with 500 ng/well of purified DerP or MBP-ProDerP1 and inculated with 95 sera (diluted 1:8) radioallergosorbent positive to D. pteronyssinus. Bound IgE was quantitated by incubation with mouse anti-human IgE and alkaline phosphatase-labelled anti-mouse Ig antibodies, followed by an enzymatic assay. Results are expressed as OD_{410nm} values.

Figure 3: IgE-binding reactivities of MBP-ProDerP1 mutants, carrying the mutations C4R, C31R and C65R. Immunoplates were coated with 500ng/well of Wild-type or mutant MBP-ProDerP1 and incubated with a pool of 20 sera (diluted 1:8) radioallergosorbent positive to *D. pteronyssinus*. Bound IgE was quantitated by incubation with mouse anti-human IgE and alkaline phosphatase-labelled anti-mouse IgG antibodies, followed by an enzymatic assay. Results are expressed as OD_{410nm} values.

Figure 4: Histamine release activity of allergens. Basophils isolated from the peripheral blood of one allergic donor were stimulated with serial dilutions of diiferent allergens. The histamine released from cells was measured by ELISA. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630. Results are shown as the ratio of released histamine by allergens to total histamine.

Figure 5: schematic representation of the animal model of house dust mite allergy.

The examples which follow are illustrative but not limiting of the invention. Restriction enzymes and other reagents were used substantially in accordance with the vendors' instructions.

EXAMPLE I

General procedures

1. - SDS PAGE and Western blot analysis

Proteins were analyzed by SDS-PAGE on 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes using a semi-dry transblot system (Bio-Rad). Membranes were saturated for 30 min with 0.5% Instagel (PB Gelatins) in TBS-T (50mM Tris HCl pH 7.5, 150mM NaCl, 0.1% Tween 80) and incubated with mouse polyclonal serum raised against denatured or native ProDerP1 diluted in blocking solution (1: 5000). Immunoreactive materials were detected using alkaline phosphatase-conjugated goat anti-mouse antibodies (Promega, 1:7500) and 5-bromo,4-chloro,3-indolylphosphate (BCIP, Boehringer)/ nitroblue tetrazolium (NBT, Sigma) as substrates.

2. - Glycan analysis

Carbohydrate analysis was carried out with the Glycan Differenciation Kit (Boehringer) using the following lectins: *Galanthus nivalis* agglutinin (GNA), *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* agglutinin (MAA), Peanut agglutinin (PNA) and *Datura stramonium* agglutinin (DSA). Briefly, purified proteins were transferred from SDS-PAGE onto nitrocellulose membranes. Membranes were incubated with the different lectins conjugated to digoxigenin. Complexes were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase.

3. - Enzymatic assays

Enzymatic assays were performed in 50 mM Tris-HCl pH 7, containing 1mM EDTA and 20mM L-cysteine at 25°C in a total volume of 1ml. Hydrolysis of Cbz-Phe-Arg-7-amino-4-methylcoumarin (Cbz-Phe-Arg-AMC) and Boc-Gln-Ala-Arg-7-amino-4-

methylcoumarin (Boc-Gln-Ala-Arg-AMC) (Sigma) (both substrates at a final concentration of 100μM) was monitored using a SLM 8000 spectrofluorimeter with λ_{ex} = 380nm and λ_{em} = 460nm. Assays were started by addition of cysteine activated allergen to a final concentration of 100 nM. Before any assay, purified DerP1 or ProDerP1 was incubated with a mixture of aprotinin- and p-aminobenzamidine-agarose resins (Sigma) to remove any putative trace of serine protease activity.

4. - Protein determination

Total protein concentration was determined by the bicinchoninic acid procedure (MicroBCA, Pierce) with bovine serum albumin as standard.

5. - DerP1 ELISA

DerP1 or recProDerP1 was detected with an ELISA kit using DerP1 specific monoclonal antibodies 5H8 and 4C1 (Indoor Biotechnologies). The DerP1 standard (UVA 93/03) used in the assay was at a concentration of 2.5μg/ml.

6. - IgE-binding activity

Immunoplates were coated overnight with DerP1 or ProDerP1 (500ng/well) at 4°C. Plates were then washed 5 times with 100μl per well of TBS-Tween buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 80) and saturated for 1 hr at 37°C with 150μl of the same buffer supplemented with 1% BSA. Sera from allergic patients to *D. pteronyssinus* and diluted at 1/8 were then incubated for 1 hr at 37°C. Out of the 95 sera used in the experiments, 16 sera ranged in their specific anti-*D. pteronyssinus* IgE values (RAST assays) from 58.1kU/L to 99kU/L and 79 above the upper cut-off value of 100kU/L. Plates were washed 5 times with TBS-Tween buffer and the allergen-IgE complexes were detected after incubation with a mouse anti-human IgE antibody (Southern Biotechnology Associates) and a goat anti-mouse IgG antibody coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer, Promega). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{410nm} was measured in a Biorad Novapath ELISA reader.

For IgE inhibition assays, plates were coated with DerP1 or ProDerP1 at the same concentration (0.12 μM). A pool of 20 human sera from allergic patients (RAST value >

100kU/L) was preincubated overnight at 4°C with various concentrations (3.6-0.002 µM) of DerP1 or recProDerP1 as inhibitors and added on ELISA plates. IgE-binding was detected as described above.

7. - Histamine release

The histamine release was assayed using leukocytes from the peripheral heparinized blood of an allergic donor and by the Histamine-ELISA kit (Immunotech). Basophils were incubated with serial dilutions of recProDerP1 or DerP1 for 30min at 37°C. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630 (Sigma).

8. – ProDerP1 denaturation

Recombinant ProDerP1 was heat-denatured for 5 min at 100°C in presence of 50mM β-mercaptoethanol.

9. - Immunisations

Groups of ten CBA/J mice (six weeks old) were four weekly immunised with 5µg of different proteins or 100µg of different plasmidic DNA. The purified allergens were injected in presence of alum as adjuvant. As controls, groups of mice were immunised with alum or pJW4304 DNA vector. Mice were bled from the retro-orbital venous plexus on days 7, 14, 21, 28 and sera were collected.

10. - Bronchoprovocation

Within 72h after immunisations, all mice were placed in a Plexiglas chamber (13 x 19 x 37.5 cm) and exposed to aerosolised crude *D.pteronyssinus* extract over a 20-min period for 7 consecutive days. The concentration of crude mite extract was 300µg/ml. The aerosols were generated by an ultrasonic nebulizer (Syst' AM). The output of the nebulizer was 0.5ml/min and the mean particle size of the aerosol was between 1 and 5 µm. As control, mice were nebulized with PBS.

11. - Measurement of DerP1-specific IgG, IgG1 and IgG2a

Sera were assayed for anti-DerP1 IgG, IgG1 and IgG2a antibodies by ELISA. Immunoplates were coated with ProDerP1 (500ng/well), for 16 hrs at 4°C. Plates were washed 5 times with TBS-Tween (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 80) and saturated for 1 hr at 37°C with 150µl of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 hr at 37°C. Plates were washed 5 times with TBS-Tween buffer and antigen-bound antibodies were detected with the second antibody (goat anti-mouse IgG, Promega, USA) coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Biorad Novapath ELISA reader.

Mouse antibody subclass was determined using immunoplates coated as described above and IgG1- or IgG2a-specific biotin-labelled monoclonal antibodies (rat anti-mouse, dilution 1/7000 in TBS-Tween buffer and 1% BSA, Biosource) as second antibodies. Phosphatase alkaline-conjugated streptavidin (1/1000 dilution, Amersham) was added to each well. Assay of the enzymatic activity proceeded as described above.

In all cases, ELISA titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal $O.D._{415}$ value.

12. - Measurement of DerP1-specific IgE

Immunoplates were coated with rat anti-mouse IgE (10ng/well), for 16 hrs at 4°C. Plates were washed 5 times with TBS-Tween (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 80) and saturated for 1 hr at 37°C with 150µl of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 hr at 37°C. ProDerP1 was then added at 500ng/ml in saturation buffer. Bound ProDerP1 was detected by addition of biotinylated anti-DerP1 monoclonal antibody 4C1 (Indoor Biotechnologies) Plates were washed 5 times with TBS-Tween buffer and antibodies-bound antigen were detected with addition of streptavidin coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Biorad Novapath ELISA reader.

13. - Proliferation assays

To measure DerP1-specific T-cell proliferative response, immunised mice were sacrificed before and after bronchoprovocations. Lymphocytes were isolated from spleens. Cells (4 x 10^5/well in triplicate), cultured in RPMI 1640 with 10% FCS containing 15mM HEPES and 30μM β-mercaptoethanol, were stimulated with serial dilutions of crude mite extract or ProDerP1 in 96-well plates (10 base 2 dilutions of the antigen were tested, starting from a concentration of 25μg/ml). As control, cells were incubated with only RPMI medium. After 4 days, cells were pulsed with 1μCi/well [^{3}H] thymidine (Amersham) for 16 hours. Cells were harvested and ^{3}H-thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the means of quadruplicate wells and were expressed as stimulation index (SI). A stimulation index of > 2 was considered positive.

14. - Cytokines assay

The level of IFNγ and IL-5 in the lymphocyte culture supernatants were measured in ELISA assays. Plates were coated with 1μg/ml of anti-mouse IL-5 monoclonal (PharMingen) or anti-mouse IFNγ (Biosource) polyclonal antibodies. Plates were washed 5 times with TBS-Tween and saturated for 1 hr at 37°C with 150μl of TBS-Tween-BSA. Serial dilutions of splenocyte culture supernatants were added and incubated for 90 min at 37°C. Biotinylated anti-mouse IL-5 (PharMingen, 1μg/ml) or anti-mouse IFNγ (Biosource, 0.2μg/ml) antibodies were applied to the plates for 1h at 37°C. The antigen-antibody complexes were detected by incubation with streptavidin coupled to horseradish peroxydase (dilution 1/10000, Amersham). The enzymatic activity was measured using tetramethylbenzidine (TMB) as substrate (Sigma). The absorbance at 460nm was measured in a Biorad Novapath ELISA reader. Cytokine concentrations were determined by interpolation from a standard curve performed with purified mouse IL-5 or IFNγ.

15. - Bronchoalveolar lavage

Three days after the final aerosol exposure, mice were bled and sacrificed. The lungs were immediately washed via the trachea cannula with 1ml Hank's balanced salt solution (HBSS) which was instilled and gently recovered by aspiration three times. The lavage

fluid was centrifuged at 400g for 10min at 4°C. The cell pellet was resuspended in 300µl Hank's balanced salt solution (HBSS) and cells were counted in a Thoma hemocytometer. Cytospin preparations from 50µl-aliquots were stained with May-Grünwald Giemsa 's stain for differential cell counts.

EXAMPLE II

Expression of MBP-ProDerP1 in *E. coli*

1. - Construction of MBP-ProDerP1 expression vector

The complete synthetic cDNA encoding ProDerP1 (1-302 aa) (SEQ ID NO:1) was isolated from the eukaryotic expression plasmid pNIV 4846 (a pEE 14-derived expression plasmid carrying humanized ProDerP1 coding cassette, (M.Massaer *et al.*, International Archives of Allergy and Immunology, 2001, **125**:32-43) after digestions with *Eag* I and *Xba* I. DNA was blunted using large fragment DNA polymerase (Klenow) before *Xba* I restriction. The 921 bp fragment was inserted at the *Asp* 718 (blunted end)-*Xba* I site of pMAL-c2E (New England Biolabs) to give pNIV4854, downstream of the MBP gene. The amino acid sequence of ProDerP1, encoded by the cDNA of SEQ ID NO:1, is represented in fugure 2 (SEQ ID NO:2).

2. – Site-directed mutagenesis

Mutagenesis of DerP1 cysteine residues at position 4, 31 or 65 (mature ProDerP1 numbering, corresponds to positions 84, 111 or 145 in ProDerP1) was performed in the plasmid pNIV4854, after the substitution of DNA fragments carrying one of the three cysteine codons by synthetic oligonucleotides containing the mutations. The following oligonucleotides were used:

5'TTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCCGTATCAACGGCA ATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGGACCGTGACTCCCATCCG CATGC3' (forward) and 5'CGGATGGGAGTCACGGTCCTCATCTG GCGCAGATCAATCTCAGCGGGGGCATTGCCGTTGATACTACGGGCGTTGGTC TCCGCGTTGAGATCGAAACTGGGTC3' (reverse) to generate a 110bp *Afl* II-*Sph* I fragment for the mutation of cysteine residue 4 to arginine (C4R),

5'CAAGGCGGCCGTGGGTCTTGTTGGGCCTTTTCAGGCGTGGCCGCGACAG AGTCGGCATACCTCGCGTATCGGAATCAGAGCCTGGACCTCGC3' (forward) and 5'TCAGCGAGGTCCAGG CTCTGATTCCGATACGCGAGGTATGCCGACT CTGTCGCGGCCACGCCTGAAAAGGCCCAACAAGACCCACGGCCGCCTTGCAT G3' (reverse) to generate a 98bp *Sph* I-*Blp* I fragment for the mutation of cysteine residue 31 to arginine (C31R), 5'TGAGCAGGAGCTCGTTGACCGTGCCTCC CAACACGGATGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGC ATA3' (forward) and 5'CTGGATGTATTCGATACCTCTGGGAATCGTAT CC CCCATGACATCCGTGTTGGGAGGCACGGTCAACGCGCTCCTGC3' (reverse) to generate a 82bp *Afl* II-*Sph* I fragment for the mutation of cysteine residue 65 to arginine (C65R).

The resulting plasmids containing the ProDerP1 cassette downstream to the MBP gene and carrying respectively the mutations C4R, C31R and C65R were called pNIV4870, pNIV4871 and pNIV4872. All the three mutations were verified by DNA sequencing. Mutated ProDerP1 amino acid sequences respectively carrying C4R, C31R and C65R mutation are illustrated in SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7 respectively. The corresponding encoding nucleic acid sequences are shown in SEQ ID NO:4 (C4R mutation), SEQ ID NO:6 (C31R mutation) and SEQ ID NO:8 (C65R mutation).

3. – Expression and purification of wild-type and mutant MBP-ProDerP1

E. coli containing the different recombinant expression vectors were grown overnight at 37°C in 869 medium (A.Jacquet *et al.*, Prot. Exp. Purif. 1999, **17**, 392-400) with 100 µg/ml ampicillin. Cells were then diluted 1:100 and allowed to grow at 37°C to an optical density between 0.4 and 0.6 at 600 nm. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.3 mM. After a 2h period of induction, cells were harvested by centrifugation at 10000 rpm for 15min.

Bacterial cell pellets from 1 liter cultures were resuspended in 20mM Tris-HCl pH 7.5, containing 1mM aprotinin and AEBSF, and broken under a pressure of 1800 bars using a Cell disrupter (Constant Systems Ltd, Warwick, UK). The lysate was ultracentrifugated at 150,000g for 60 min. The pellet resulting from the ultracentrifugation was washed with 20mM Tris-HCl pH 7.5. Insoluble proteins were extracted overnight at 4°C with 20mM Tris-HCl pH 7.5 containing 6M urea. The suspension was ultracentrifugated at 150,000g

for 60 min. The supernatant was directly dialysed overnight against 20mM Tris-HCl pH 7.5, 200mM NaCl, 1mM EDTA. The solution was centrifugated to remove any precipitated protein and directly applied onto an amylose resin (1 x 15 cm) equilibrated in the same buffer. The column was washed with the starting buffer until the A_{280nm} reached the baseline. Proteins were eluted by the addition of 10mM maltose in the column buffer. Fractions containing the fusion proteins were pooled and concentrated. Purified proteins were stored at –20°C.

EXAMPLE III

Expression of three different ProDerP1 mutants in CHO cells

1. – Site-directed mutagenesis

Mutations of DerP1 cysteine residues at position 4, 31 or 65 (mature DerP1 numbering, corresponds to positions 84, 111 or 145 in ProDerP1) were introduced into the plasmid pNIV4846. Plasmids pNIV4870, pNIV4871 and pNIV4872, containing the DerP1 cassette downstream to the MBP (see Example II) gene and carrying respectively the mutations C4R, C31R and C65R were each restricted with *Sfu*I-*Xho*I to isolate a 714bp fragment. The purified DNA fragments were inserted into plasmid p4846 previously cleaved with the same restriction enzymes. The resulting plasmids containing the DerP1 variants C4R, C31R and C65R were called pNIV4873, pNIV4875 and pNIV4874.

2. – Transient transfections and selection of ProDerP1-producing stable CHO-K1 lines.

To determine the production of DerP1 by plasmids pNIV4873, pNIV4875 and pNIV4874, COS cells were transiently transfected by lipofection. For stable DerP1 expression, CHO-K1 cells were transfected with the different plasmids by lipofection. After a 3-weeks 25µM methionylsulphoximin (MSX) selection, one round of gene amplification was carried out with 100µM MSX.

EXAMPLE IV

Denatured ProDerP1 displays IgG but not IgE-binding reactivity towards allergic sera.

To determine whether a denatured form of ProDerP1 could be used as a hypoallergenic vaccine, IgG- and IgE binding reactivities of denatured (5 min at 100°C in the presence of 50mM β-mercaptoethanol) ProDerP1 were assayed in ELISA tests. As shown in figure 1, denatured ProDerP1 conserved the main part of the IgG epitopes present on native ProDerP1. On the other hand, the denatured allergen highly lost its IgE-binding reactivity. Our data suggest that denatured ProDerP1 could represent a hypoallergenic variant of ProDerP1.

EXAMPLE V

IgE reactivities of MBP-ProDerP1.

The aim of the experiment was to compare the IgE reactivity of MBP-ProDerP1 and of natural DerP1. The reactivity of MBP-ProDerP1 with specific IgE from sera of allergic patients was assessed in a direct ELISA wherein immunoplates were directly coated with DerP1 or MBP-ProDerP1. Figure 2 shows a strong correlation between the IgE binding to DerP1 and MBP-ProDerP1.

EXAMPLE VI

IgE-binding reactivities of MBP-ProDerP1 mutants.

The IgE-binding capacity of MBP-ProDerP1 mutants was determined in direct ELISA assays for which immunoplates were directly coated with the different forms of MBP-ProDerP1. A serum pool, made from 20 individual *D. pteronyssinus*-allergic patient sera with RAST value >100 kU/L, were used in the assays. As shown in figure 3, the IgE binding reactivity of the variants C31R and C65R drastically decreased to 5% compared with that of wild-type MBP-ProDerP1. Strikingly, no reactivity (0% left) of IgE to MBP-

ProDerP1 was observed when residue cysteine 4 was mutated to arginine. The IgE reactivities were specific of the ProDerP1 moiety as there were no IgE-mediated immune recognitions of MBP or MBP in fusion with an irrelevant protein. Similar results were obtained with another serum pool from 20 others patients.

5

EXAMPLE VII

Histamine release activity of various forms of ProDerP1.

10 To compare the allergenic activity of natural DerP1 with that of recombinant mutated derivatives of ProDerP1, basophils from one allergic patient were challenged *in vitro* with various concentrations of allergens and the released histamine was measured. As shown in figure 4, natural DerP1 was able to induce histamine release from basophils even at a concentration of 1ng/ml. By contrast, recombinant mutated forms of ProDerP1 could only
15 release histamine at a 1000-10000-fold higher concentration, These results clearly showed that ProDerP1 mutants display lower IgE binding reactivity than does the natural DerP1.

20 **EXAMPLE VIII**

Immunogenicity experiments with various forms of ProDerP1.

1. – Animal model of house dust mite allergy

An animal model of house dust mite allergy has been developed. CBA/J mice were
25 injected with purified DerP1 adjuvanted with alum. After four injections at one week interval, animals were subjected to a series of bronchoprovocation with *D. pteronyssinus* extract (figure 5). This model was used to test different recombinant forms of DerP1 as well as different DNA as prophylactic vaccines against house dust mite allergy.

2. – Vaccine formulations

Table 1 : protein and DNA vaccine formulations tested in the house dust mite allergy animal model depicted in figure 5.

Protein	DNA	Adjuvant	Way of injection
Natural DerP1		Alum	IP
ProDerP1 native		Alum	IP
ProDerP1 native		—	IM
ProDerP1 denatured		Alum	IP
MBP-ProDerP1		Alum	IP
MBP-ProDerP1 C4R		Alum	IP
MBP-ProDerP1 C31R		Alum	IP
MBP-ProDerP1 C65R		Alum	IP

IP= intraperitoneal injection

IM=intramuscular injection

3. – Antibody response - Results

Mice immunized by four injections of natural DerP1 produced high titers of IgG and IgG1, low titers of IgG2a and large amounts of IgE antibodies, indicating that natural DerP1 induces strong Th2 immunes responses (Tables 2 and 4).

The anti-DerP1 IgG and IgG1 antibody responses were also strong in mice injected with native or denatured ProDerP1. After injections with native ProDerP1, the IgG2a titers were slightly higher than those obtained with DerP1, IgE titers being comparable or slightly lower than those obtained with DerP1. In contrast to the native ProDerP1-immunized mice, animals injected with denatured ProDerP1 produced high IgG2a titers and very low IgE antibodies. As expected, immunizations with ProDerP1 in the absence of Alum induced poor immune responses (Table 4).

MBP-ProDerP1 wild type (WT), C4R, C31R and C65R-sensitized mice showed similar productions of specific IgG and IgG1 antibodies (Table 3). Highest IgG2a titers were observed in groups immunized with MBP-ProDerP1 WT and C31R.

Specific IgE titers were low, whatever the MBP-ProDerP1 variants injected.

Similar results were obtained after mice immunizations with plasmid encoding ProDerP1.

Table 2 : Titers of specific anti-DerP1 antibodies from mice immunized with different antigens. For IgE titers, results are expressed as OD_{415nm} values for a 1/10 dilution of sera. Titers were also measured after bronchoprovocations with PBS or with *D. pteronyssinus* extracts (HDM).

Antigen	Bleeding	Challenge	IgG	IgG1	IgG2a	IgE
DerP1	1		< 50	< 50	< 50	0
	2		214	900	< 50	1.1
	3		700	6062	< 50	0.2
	4		2500	24390	100	0.6
	5	PBS	8670	16340	300	0.7
		HDM	8230	17440	300	0.6
ProDerP1 native	1		< 50	< 50	< 50	0
	2		301	1146	< 50	1.1
	3		800	6860	86	0.3
	4		2500	28545	203	0.5
	5	PBS	8266	25500	600	0.3
		HDM	11880	38310	600	0.6
denatured	1		< 50	< 50	< 50	0
	2		330	861	120	0.2
	3		966	3402	210	0.07
	4		3093	14830	970	0.1
	5	PBS	16380	54040	2700	0.1
		HDM	14200	32140	2700	0.05

Table 3 : Titers of specific anti-DerP1 antibodies from mice immunized with different antigens. For IgE titers, results are expressed as OD_{415nm} values for a 1/10 dilution of sera. Titers were also measured after bronchoprovocations with PBS or with *D. pteronyssinus* extracts (HDM).

Antigen	Bleeding	Challenge	IgG	IgG1	IgG2a	IgE
MBP-ProDerP1 WT	2		637	3351	144	0.046
	3		4444	24720	757	0.039
	4		2500	24390	100	0.6
	5	PBS	6151	29500	2899	0,13
		HDM	3437	22210	1496	0,27
MBP-ProDerP1 C4R	2		583	2212	95	0
	3		1123	6131	356	0.021
	4		2500	28545	203	0.5
	5	PBS	2064	9077	624	0,004
		HDM	2418	14390	635	0,029
MBP-ProDerP1 C31R	2		1221	4572	144	0.017
	3		6472	40405	1311	0.029
	4		3093	14830	970	0.1
	5	PBS	2897	10880	857	0,063
		HDM	5508	24300	1959	0,074
MBP-ProDerP1 C65R	2		202	887	< 50	0.022
	3		1252	5718	363	0.066
	4		3093	14830	970	0.1
	5	PBS	782	3958	87	0,108
		HDM	3109	16250	430	0,117

Table 4: Titers of specific anti-DerP1 antibodies from mice immunized with different antigens. For IgE titers, results are expressed as OD_{415nm} values for a 1/10 dilution of sera. Titers were also measured after bronchoprovocations with PBS or with *D. pteronyssinus* extracts (HDM).

Antigen	Bleeding	Challenge	IgG	IgG1	IgG2a	IgE
DerP1	2		201	1135	< 20	0.852
	3		3264	18002	< 50	0.34
	4		8271	43306	< 50	0.59
	5	PBS	10072	57670	< 100	0.44
		HDM	6058	72810	< 100	0.68
ProDerP1 Alum	2		929	7422	159	0.8
	3		5061	27244	586	0.37
	4		15110	68960	1016	0.46
	5	PBS	10900	57255	1190	0,421
		HDM	16770	79460	1125	0,485
ProDerP1 (no adjuvant)	2		136	774	< 20	0.58
	3		1389	8571	104	0.13
	4		4704	14126	120	0.17
	5	PBS	3587	16930	105	0.28
		HDM	3880	20737	100	0.25

4. - T-cell proliferative response - Results

Before (control) and after aerosol challenge, splenocytes isolated from immunized mice were examined for T-cell proliferative response by stimulation with ProDerP1 or *D. pteronyssinus* extract. Results are shown in Table 5 (stimulation index) and in Table 6 (cytokines).

Allergen-specific T cell responses were detected in immunized mice with the different recombinant ProDerP1 mutants. Strongest responses were observed when splenocytes were restimulated with ProDerP1. T-cell reactivities appeared to be independent from the challenge.

These results in Table 5 indicated that the different forms of ProDerP1 shared common T-cell epitopes with natural DerP1. Moreover, destructuration of ProDerP1 by thermal denaturation or site-directed mutagenesis did not alter ProDerP1 T-cell reactivity,

confirming that these forms are hypoallergens with very low IgE-binding reactivity able to stimulated T-cell responses.

Table 5:

Vaccinated mice were challenged or not with PBS or *D. pteronyssinus* extracts. Spleen cells were isolated and restimulated in vitro with purified ProDerP1 or with *D. pteronyssinus* extracts. Stimulation index was measured by [^{3}H]-thymidine incorporation. -: not available. These results are obtained from different experiments, not from only one. Consequently, cytokine assays can not be compared between all groups.

Antigen	Concentration of stimulating antigen (µg/ml)	S.I. (stimul. with ProDerP1)			S.I. (stimul. with HDM ext.)		
		aerosol			aerosol		
		None	PBS	HDM	None	PBS	HDM
MBP-ProDerP1 WT	50	7.3	14.97	20.8	-	-	-
MBP-ProDerP1 C4R	50	19.1	9.7	16.3	-	-	-
MBP-ProDerP1 C31R	50	5.4	10.0	14.7	-	-	-
MBP-ProDerP1 C65R	50	6.8	8.8	13.0	-	-	-
DerP1	40	-	1.6	17.5	-	1.6	7.5
ProDerP1	40	-	30.9	11.5	-	2.8	2.8
ProDerP1 denatured	40	-	24.0	15.9	-	1.7	1.4
Alum	40	-	4.2	4.6	-	2.0	1.3

The presence of cytokines IL-5 and IFNγ in the culture supernatants of restimulated splenocytes was determined in ELISA (Table 6). If we compared the ratio [IFNγ]/[IL-5], we could conclude that vaccinations with natural DerP1 or ProDerP1 adjuvanted with alum induced a better production of IL-5 than IFNγ. The different forms of MBP-ProDerP1 (mutants and wild-type) as well as denatured ProDerP1 induced comparable levels of both cytokines.

Table 6: [IL-5] and [IFNγ] in supernatants from ProDerP1-restimulated splenocytes. These results are obtained from different experiments, not from only one. Consequently, cytokine assays can not be compared between all groups.

Antigen	[IL-5] (pg/ml)			[IFNγ] (pg/ml)		
	Aerosol			Aerosol		
	none	PBS	HDM	None	PBS	HDM
MBP-ProDerP1	420	165	929	987	1076	1282
MBP-ProDerP1C4R	330	51	308	551	1366	1177
MBP-ProDerP1C31R	430	202	1141	1348	1281	3392
MBP-ProDerP1C65R	0	0	953	0	0	1161
Alum	0	0	0	0	0	0
DerP1	75	45	495	0	0	190
ProDerP1	0	355	400	0	125	210
ProDerP1 denatured	-	850	736	-	822	1119

5. – Bronchoalveolar lavage - Results

Sensitisation with natural DerP1 and subsequent exposure to aerosolised house dust mite extracts induced significantly higher bronchoalveolar cell numbers (Table 7). Seven exposures to aerosolised house dust mite extracts were shown to induce airway eosinophilia in only the animals vaccinated with DerP1. In this group, airway eosinophilia was not observed when DerP1-sensitised animals were not nebulized or exposed to aerosolised PBS.

Vaccinations with the different recombinant forms of ProDerP1 prevented airway eosinophilia, even after exposure to aerosolised HDM extracts.

Table 7: Characterization of the bronchoalveolar lavage fluid of different antigen-immunized mice exposed to PBS or house dust mite extracts aerosols

Antigen	Aerosol	Lympho (%)	Eosino (%)	Neutro (%)	Macro (%)	Mono (%)	Total cells (10^5/ml)
DerP1	none	86	4	0	6	3	2.2
	HDM	13	68	7	6	6	167
	PBS	90	0	2	4	4	4.8
ProDerP1	none	90	0	0	7	3	3.2
	HDM	69	7	12	3	10	5.1
	PBS	76	5	4	7	8	7.6
ProDerP1 denatured	none	51	5	2	22	20	4
	HDM	52	4	26	10	7	6.9
	PBS	67	2	2	20	9	5.2
Alum	none	88	1	4	7	0	3.6
	HDM	80	0	4	14	1	1.5
	PBS	88	1	5	5	1	1.2
MBP-ProDerP1	none	85	2	4	7	0	1.5
	HDM	70	3	14	8	5	2.1
	PBS	88	1	6	5	0	0.6
MBP-ProDerP1 C4R	none	90	2	4	4	1	2.2
	HDM	71	2	14	11	1	2
	PBS	80	2	7	10	1	4.5
MBP-ProDerP1 C31R	none	79	1	14	7	0	1.3
	HDM	65	4	27	5	1	2
	PBS	87	2	7	5	1	3
MBP-ProDerP1 C65R	none	85	0	4	10	1	2.4
	HDM	84	1	7	7	1	2.4
	PBS	84	1	4	12	0	1.5

EXAMPLE IX

Expression plasmid for nucleic acid vaccination (NAVAC)

1. - Construction of ProDerP1 encoding plasmid for nucleic acid vaccination

The ProDerP1 coding cassette (1-302aa) was excised from plasmid pNIV4846 (see above), restricted with *Hind*III and *Bgl*II, and inserted into plasmid pJW4304 previously cleaved with *Hind*III and *Bgl*II. The resulting plasmid, named pNIV4868, was verified by DNA sequencing.

2. – Site-directed mutagenesis

Mutations of ProDerP1 cysteine residues at position 4, 31 or 65 (mature DerP1 numbering, corresponds to positions 84, 111 or 145 in ProDerP1) were introduced into the plasmid pNIV4868. Plasmids pNIV4870, pNIV4871 and pNIV4872, containing the ProDerP1 cassette downstream to the MBP gene and carrying respectively the mutations C4R, C31R and C65R were each restricted with *Afl*II-*Bam*HI to isolate a 699bp fragment. pNIV 4868 was digested with *Afl*II-*Hpa*I to isolate a 480bp fragment. The two purified DNA fragments were inserted into plasmid pJW4304 previously cleaved with *Hpa*I-*Bam*HI. The resulting plasmids containing the ProDerP1 variants C4R, C31R and C65R were called pNIV4879, pNIV4880 and pNIV4881.

EXAMPLE X

Expression of ProDerP1 in *Pichia pastoris*

1. - Construction of ProDerP1 expression vector

The ProDerP1 coding cassette from pNIV4846 (full-length 1-302aa ProDerP1 cDNA with optimised mammalian codon usage) was amplified by PCR using the following primers: 5'ACTGAC**AGGCCT**CGGCCGAGCTCCATTAA3' (*Stu*I restriction site in bold, forward) and 5'CAGTCA**CCTAGG**TCTAGACTC GAGGGGAT3' (*Avr*II restriction site in bold, reverse). The amplified fragment was cloned into the pCR2.1 TOPO cloning vector. The correct ProDerP1 cassette was verified by DNA sequencing. Recombinant TOPO vector was digested with *Stu*I-*Avr*II to generate a 918bp fragment

which was introduced into the pPIC9K expression vector restricted with *Sna*BI-*Avr*II. The resulting plasmid, pNIV4878, contains the ProDerP1 cassette downstream to the S.cerevisae α factor

2. - Site-directed mutagenesis

Expression plasmid for the production of unglycosylated ProDerP1 (N52Q, mature DerP1 numbering) was derived from pNIV4878 by overlap extension PCR using a set of four primers. The following primers:

5'GGCTTTCGAACAC**CTTAAG**ACCCAG3' (primer 1, *Afl*II restriction site in bold, forward) and 5'GCTCCCTAGC**TACGTA** TCGGTAATAGC3' (primer 2, *Sna*BI restriction site in bold, reverse) were used to amplify a 317bp fragment encoding the ProDerP1 amino acid sequence 71-176.

The following primers 5'CCTCGCGTATCGG**CAA**CAGAGCCTGGACC3' (primer 3, mutation N52Q in bold, forward) and 5'GGTCCAGGCTCTG**TTG**CC GATACGCGAGG3' (primer 4, mutation N52Q in bold, reverse) were used to introduce mutation N52Q in the ProDerP1 sequence.

The mutated 317bp *Afl*II-*Sna*BI fragment was generated by a three-step process. In PCR n°1, primers 1 and 4 were mixed with pNIV4878 to produce a ~ 200 bp fragment. In PCR n°2, primers 2 and 3 were mixed with pNIV4878 to produce a ~ 140 bp. The two PCR products were purified onto agarose gel and used as templates for a third round of PCR to obtain a ~ 340 bp fragment. This purified fragment was cloned into the pCR2.1 TOPO cloning vector (Invitrogen). The mutation was verified by DNA sequencing. Recombinant TOPO vector was digested with *Afl*II-*Sna*BI to generate a 317bp fragment which was ligated into the similarly digested pNIV4878. The resulting plasmid, pNIV4883, contains the ProDerP1 N52Q downstream to the S.cerevisae α factor.

To obtain unglycosylated variants of ProDerP1 carrying mutations of DerP1 cysteine residues at position 4, 31 or 65 (mature DerP1 numbering), overlap extension PCR using the same set of primers were performed with plasmids pNIV4873, pNIV4875 and pNIV4874. The resulting plasmids pNIV4884, 4885 and 4886 encode respectively ProDerP1 N52Q C4R, N52Q C31R and N52Q C65R.

2. - Transformation of *P. pastoris*

Plasmid pNIV4878 was introduced *into P. pastoris* using the spheroplast transformation method. Transformants were selected for histidinol deshydrogenase (His+) prototrophy. The screening of His+ transformants for geneticin (G418) resistance was performed by plating clones on agar containing increasing concentrations of G418.

Transformation with plasmids encoding ProDerP1 N52Q, ProDerP1 N52Q C4R, N52Q C31R and N52Q C65R was performed using the same method.

3. - Production of ProDerP1 by recombinant yeast

G418 resistant clones were grown at 30°C in BMG medium to an OD_{600nm} of 2-6. Cells were collected by centrifugation and resuspended to an OD_{600nm} of 1 in 100ml of BMG medium. ProDerP1 expression was induced by daily addition of methanol 0.5% for 6 days. The supernatant was collected by centrifugation and stored at –20°C until purification.

4. - Purification of ProDerP1 from yeast culture supernatant

Supernatants were diluted 10 times with water and, after pH adjustment to 9, directly loaded onto a Q sepharose column equilibrated in in 20mM Tris-HCl pH 9. The column was washed with the starting buffer. Protein elutions proceeded by step-wise increasing NaCl concentration in the buffer. The ProDerP1-enriched fractions were pooled and concentrated by ultrafiltration onto a Filtron membrane (Omega serie, cut-off: 10kD). The ProDerP1 purification was achieved by a gel filtration chromatography onto a superdex-75 column (1 x 30 cm, Pharmacia) equilibrated in PBS pH 7,3. Purified ProDerP1 was concentrated and stored at –20°C.

SEQUENCE INFORMATION

SEQ ID NO:1

```
   1  CGGCCGAGCTCCATTAAGACCTTCGAGGAATACAAGAAAGCCTTCAACAA
  51  GAGCTATGCCACCTTCGAGGACGAGGAGGCCGCGCGCAAGAACTTCCTGG
 101  AAAGCGTGAAATACGTGCAGAGCAACGGCGGGGCTATAAATCACCTGTCC
 151  GACCTGTCTTTAGACGAGTTCAAGAACCGGTTCCTGATGAGCGCCGAGGC
 201  TTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCT
 251  GCAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG
 301  ACCGTGACTCCCATCCGCATGCAAGGCGGCTGCGGGTCTTGTTGGGCCTT
 351  TTCAGGCGTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGAATCAGA
 401  GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACTGCGCCTCCCAACACGGA
 451  TGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCATAATGG
 501  CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
 551  GCCGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATTGCCAGATCTAC
 601  CCCCCTAATGCCAACAAGATCAGGGAGGCCCTGGCGCAGACGCACAGCGC
 651  CATCGCTGTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCACTATG
 701  ACGGGCGCACAATCATCCAGCGCGACAACGGATATCAGCCAAACTACCAC
 751  GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT
 801  CGTGAGAAACAGTTGGGACACTAACTGGGGCGACAACGGCTACGGCTACT
 851  TCGCCGCCAACATCGACCTGATGATGATCGAGGAGTACCCGTACGTGGTG
 901  ATCCTGTAA
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SEQ ID NO:2

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Asn Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys    30
Asn Phe Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala    45
Ile Asn His Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg    60
Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe    75
Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala    90
Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile   105
Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val   120
Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr Arg Asn Gln Ser Leu   135
Asp Leu Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly   150
Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His   165
Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu   180
Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn   195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala   210
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Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys 225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val 255
Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn 270
Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala 285
Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val 300
Ile Leu 302

SEQ ID NO:3.

Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe 15
Asn Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys 30
Asn Phe Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala 45
Ile Asn His Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg 60
Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe 75
Asp Leu Asn Ala Glu Thr Asn Ala Arg Ser Ile Asn Gly Asn Ala 90
Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile 105
Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val 120
Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr Arg Asn Gln Ser Leu 135
Asp Leu Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly 150
Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His 165
Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu 180
Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn 195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala 210
Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys 225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val 255
Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn 270
Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala 285
Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val 300
Ile Leu 302

SEQ ID NO:4

1 CGGCCGAGCTCCATTAAGACCTTCGAGGAATACAAGAAAGCCTTCAACAA
51 GAGCTATGCCACCTTCGAGGACGAGGAGGCCGCGCGCAAGAACTTCCTGG
101 AAAGCGTGAAATACGTGCAGAGCAACGGCGGGGCTATAAATCACCTGTCC
151 GACCTGTCTTTAGACGAGTTCAAGAACCGGTTCCTGATGAGCGCCGAGGC
201 TTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCC
251 GTAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG

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301  ACCGTGACTCCCATCCGCATGCAAGGCGGCTGCGGGTCTTGTTGGGCCTT
351  TTCAGGCGTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGAATCAGA
401  GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACTGCGCCTCCCAACACGGA
451  TGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCATAATGG
501  CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
551  GCCGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATTGCCAGATCTAC
601  CCCCCTAATGCCAACAAGATCAGGGAGGCCCTGGCGCAGACGCACAGCGC
651  CATCGCTGTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCACTATG
701  ACGGGCGCACAATCATCCAGCGCGACAACGGATATCAGCCAAACTACCAC
751  GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT
801  CGTGAGAAACAGTTGGGACACTAACTGGGGCGACAACGGCTACGGCTACT
851  TCGCCGCCAACATCGACCTGATGATGATCGAGGAGTACCCGTACGTGGTG
901  ATCCTGTAA
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SEQ ID NO:5

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Ile Asn His Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg    60
Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe    75
Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala    90
Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile   105
Arg Met Gln Gly Gly Arg Gly Ser Cys Trp Ala Phe Ser Gly Val   120
Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr Arg Asn Gln Ser Leu   135
Asp Leu Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly   150
Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His   165
Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu   180
Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn   195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala   210
Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys   225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln   240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val   255
Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn   270
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Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val   300
Ile Leu 302
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SEQ ID NO:6

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 651  CATCGCTGTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCACTATG
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 751  GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT
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SEQ ID NO:7

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Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe    75
Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala    90
Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile   105
Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val   120
Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr Arg Asn Gln Ser Leu   135
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Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His   165
Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu   180
Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn   195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala   210
Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys   225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln   240
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Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn 270
Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala 285
Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val 301
Ile Leu 302

SEQ ID NO:8

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151 GACCTGTCTTTAGACGAGTTCAAGAACCGGTTCCTGATGAGCGCCGAGGC
201 TTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCT
251 GCAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG
301 ACCGTGACTCCCATCCGCATGCAAGGCGGCTGCGGGTCTTGTTGGGCCTT
351 TTCAGGCGTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGAATCAGA
401 GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACCGTGCCTCCCAACACGGA
451 TGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCATAATGG
501 CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
551 GCCGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATTGCCAGATCTAC
601 CCCCCTAATGCCAACAAGATCAGGGAGGCCCTGGCGCAGACGCACAGCGC
651 CATCGCTGTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCACTATG
701 ACGGGCGCACAATCATCCAGCGCGACAACGGATATCAGCCAAACTACCAC
751 GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT
801 CGTGAGAAACAGTTGGGACACTAACTGGGGCGACAACGGCTACGGCTACT
851 TCGCCGCCAACATCGACCTGATGATGATCGAGGAGTACCCGTACGTGGTG
901 ATCCTGTAA

SEQ ID NO:9.

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Ile Asn His Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg 60
Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe 75
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Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile 105
Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val 120
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Asp Leu Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly 150
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Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn 195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala 210
Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys 225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val 255
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Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val 300
Ile Leu 302

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251 GCAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG
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351 TTCAGGCGTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGAATCAGA
401 GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACTGCGCCTCCCAACACGGA
451 CGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCATAATGG
501 CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
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601 CCCCCTAATGCCAACAAGATCAGGGAGGCCCTGGCGCAGACGCACAGCGC
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901 ATCCTGTAA

SEQ ID NO:11

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Asn Phe Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala 45

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Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala    90
Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile   105
Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val   120
Ala Ala Thr Glu Ser Ala Tyr Leu Ala Tyr Arg Asn Gln Ser Leu   135
Asp Leu Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly   150
Cys His Gly Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His   165
Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu   180
Gln Ser Arg Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn   195
Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala   210
Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile Lys   225
Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln   240
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Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn   270
Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala   285
Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val   300
Ile Leu 302
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SEQ ID NO:12

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 901 ATCCTGTAA
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Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile 105
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Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn 195
Tyr Arg Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg Glu Ala 210
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Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln 240
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Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn 270
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Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val 300
Ile Leu 302

SEQ ID NO:14

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201 TTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCT
251 GCAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG
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401 GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACTGCGCCTCCCAACACGGA
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501 CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
551 GCCGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATCGTCAGATCTAC
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701 ACGGGCGCACAATCATCCAGCGCGACAACGGATATCAGCCAAACTACCAC

751 GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT

801 CGTGAGAAACAGTTGGGACACTAACTGGGGCGACAACGGCTACGGCTACT

851 TCGCCGCCAACATCGACCTGATGATGATCGAGGAGTACCCGTACGTGGTG

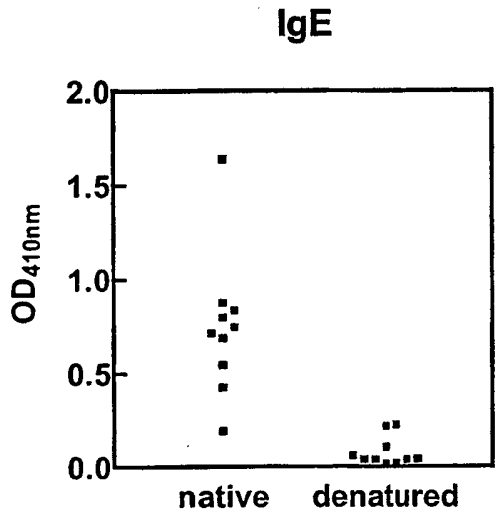
901 ATCCTGTAA

CLAIMS

1. A recombinant *Dermatophagoides pteronyssinus* DerP1 or ProDerP1 (DerP1/ProDerP1) protein allergen derivative wherein said allergen derivative has a significantly reduced allergenic activity compared to that the wild-type allergen.
2. A recombinant DerP1/ProDerP1 derivative as claimed in claim 1, wherein said derivative has been thermally treated.
3. A recombinant DerP1/ProDerP1 derivative as claimed in claim 1, wherein said derivative has been genetically mutated.
4. A recombinant DerP1/ProDerP1 mutant as claimed in claim 3, wherein said mutant comprises one or more of the DerP1 following mutation: a mutation of the cysteine 4 residue, a mutation of the cysteine 31 residue, a mutation of the cysteine 65 residue, a mutation of the cysteine 71 residue, a mutation of the cysteine 103 residue and a mutation of the cysteine 117 residue.
5. A recombinant mutant allergen having any of the sequences selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13.
6. An isolated nucleic acid molecule encoding a mutated version of an allergen as claimed in any one of claims.
7. A nucleic acid sequence according to claim 6 wherein the codon usage pattern resembles that of highly expressed mammalian genes.
8. An expression vector containing a nucleic acid of claim 6 or 7.
9. A host cell transformed with a nucleic acid sequence of claim 6 or 7 or with a vector as claimed in claim 8.
10. An immunogenic composition comprising a recombinant protein or mutant allergen as claimed in any one of claims 1 to 5, or an encoding polynucleotide as claimed in claim 6 to 8, and, optionally, an adjuvant.
11. An immunogenic composition as claimed in claim 10, wherein the adjuvant is a preferential stimulator of Th1-type immune responses.
12. An immunogenic composition as claimed in claim 10 or 11 wherein the adjuvant comprises one or more of 3D-MPL, QS21, a CpG oligonucleotide, a polyethylene ether or ester or a combination of two or more of these adjuvants.

13. An immunogenic composition as claimed in any of claims 10 to 12 wherein the allergen is presented in an oil in water or a water in oil emulsion vehicle.
14. A immunogenic composition as claimed herein for use in medicine.
15. Use of a recombinant protein or mutant allergen as claimed in any one of claims 1 to 5 in the manufacture of a medicament for the treatment of allergy.
16. A method of treating a patient suffering from or preventing a patient susceptible to allergic responses, comprising administering to said individual an immunogenic composition as claimed in claims 10 to 13.

FIG. 1: IgG and IgE-binding reactivity of denatured ProDerP1 expressed in CHO cells.



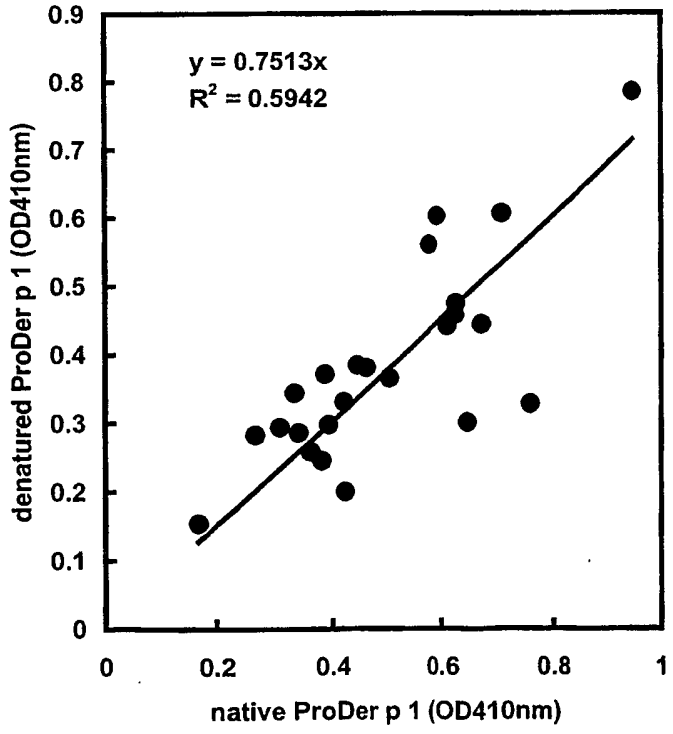


FIG. 2: Correlation between the IgE reactivity of MBP-ProDerP1 and natural DerP1.

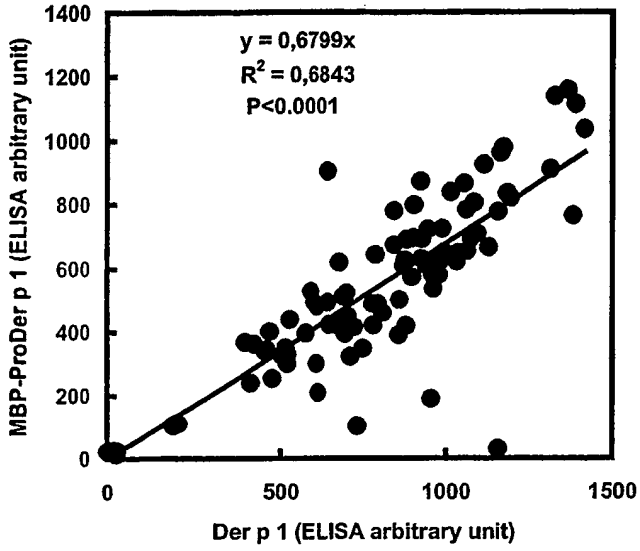


FIG. 3: IgE-binding reactivities of MBP-ProDerP1 mutants, carrying the mutations C4R, C31R and C65R.

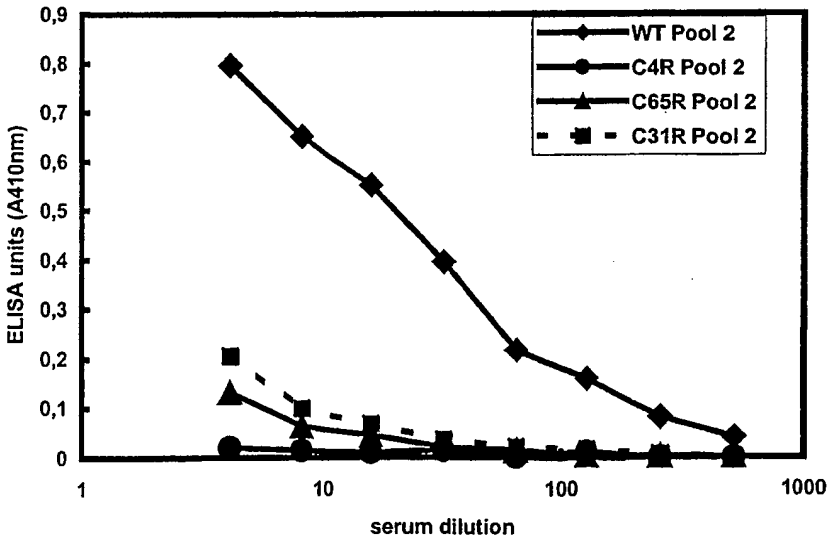


FIG. 4: Histamine release activity of allergens.

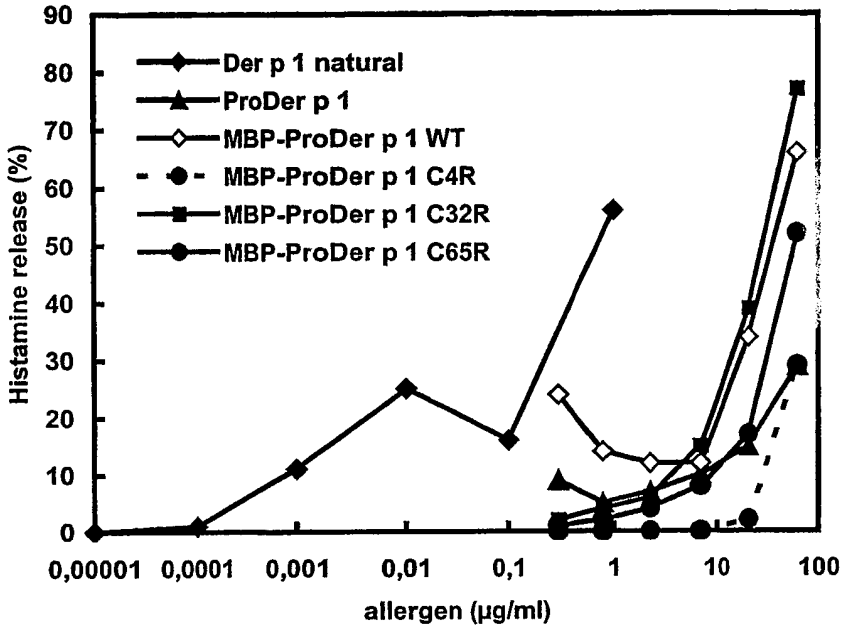
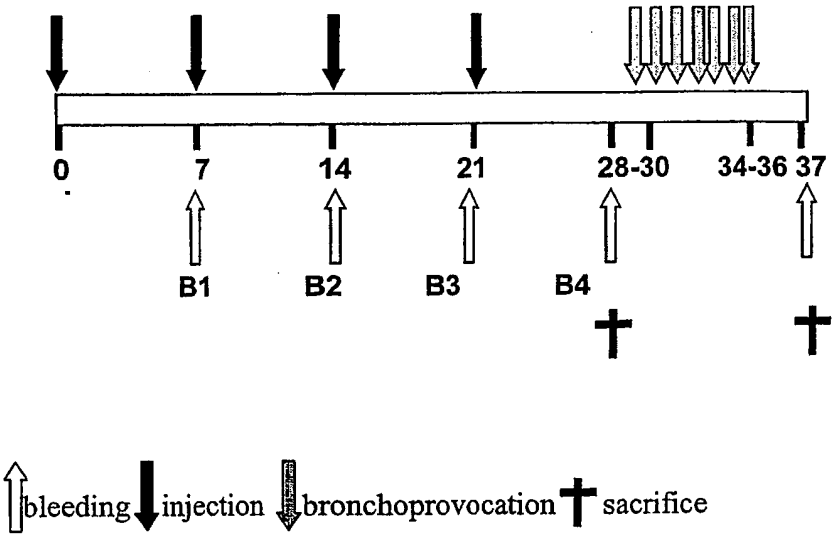


Fig. 5: Schematic representation of the animal model of house dust mite allergy.



SEQUENCE LISTING

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<130> B45282

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Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45

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Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300

taa                                                               909

<210> 2
<211> 302
<212> PRT
<213> Dermatophagoides pteronyssinus

<400> 2
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125
```

```
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
        195                 200                 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300
```

<210> 3
<211> 302
<212> PRT
<213> Artificial Sequence

<220>
<223> C4R mutant of ProDerP1

<400> 3

```
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80
Thr Asn Ala Arg Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
        195                 200                 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
```

```
225                 230                 235                 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300
```

<210> 4
<211> 909
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)...(906)

<223> C4R mutant of ProDerP1

<400> 4

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cgg ccg agc tcc att aag acc ttc gag gaa tac aag aaa gcc ttc aac   48
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15

aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc   96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
             20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
         35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
     50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
 65                  70                  75                  80

acc aac gcc cgt agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Arg Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                 85                  90                  95

cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125

gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140

tgc gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc   480
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
```

```
gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175

gta gct agg gag cag tcc tgc cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190

att tcc aat tat tgc cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
        195                 200                 205

gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220

aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300

taa                                                               909
```

<210> 5
<211> 302
<212> PRT
<213> Artificial Sequence

<220>
<223> C31R mutant of ProDerP1

```
<400> 5
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Arg Gly
            100                 105                 110
```

```
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
        195                 200                 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300
```

<210> 6
<211> 909
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)...(906)

<223> C31R mutant of ProDerP1

<400> 6

```
cgg ccg agc tcc att aag acc ttc gag gaa tac aag aaa gcc ttc aac   48
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15

aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc   96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
             20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
         35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
     50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
 65                  70                  75                  80

acc aac gcc tgc agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                 85                  90                  95
```

```
cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc cgt ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Arg Gly
            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125

gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140

tgc gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc   480
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160

gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175

gta gct agg gag cag tcc tgc cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190

att tcc aat tat tgc cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
        195                 200                 205

gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220

aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300

taa                                                               909
```

<210> 7
<211> 302
<212> PRT
<213> Artificial Sequence

<220>
<223> C65R mutant of ProDerP1

<400> 7
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
1 5 10 15
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
20 25 30
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
35 40 45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
50 55 60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65 70 75 80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
85 90 95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
100 105 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
115 120 125
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
130 135 140
Arg Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145 150 155 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
165 170 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
180 185 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
195 200 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
210 215 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225 230 235 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
245 250 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
260 265 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
275 280 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
290 295 300

<210> 8
<211> 909
<212> DNA
<213> Artificial Sequence

<220>
<221> CDS
<222> (1)...(906)

<223> C65R mutant of ProDerP1

<400> 8
cgg ccg agc tcc att aag acc ttc gag gaa tac aag aaa gcc ttc aac 48
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
1 5 10 15

aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc 96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
20 25 30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac 144

```
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
         35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
     50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
 65                  70                  75                  80

acc aac gcc tgc agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                 85                  90                  95

cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125

gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140

cgt gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc   480
Arg Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160

gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175

gta gct agg gag cag tcc tgc cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190

att tcc aat tat tgc cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
        195                 200                 205

gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220

aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285
```

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg 906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
290 295 300

taa 909

<210> 9
<211> 302
<212> PRT
<213> Artificial Sequence

<220>
<223> C71R mutant of ProDerP1

<400> 9
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
1 5 10 15
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
20 25 30
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
35 40 45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
50 55 60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65 70 75 80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
85 90 95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
100 105 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
115 120 125
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
130 135 140
Cys Ala Ser Gln His Gly Arg His Gly Asp Thr Ile Pro Arg Gly Ile
145 150 155 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
165 170 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
180 185 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
195 200 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
210 215 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225 230 235 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
245 250 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
260 265 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
275 280 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
290 295 300

<210> 10
<211> 909
<212> DNA
<213> Artificial Sequence

<220>

<221> CDS
<222> (1)...(906)

<223> C71R mutant of ProDerP1

<400> 10

```
cgg ccg agc tcc att aag acc ttc gag gaa tac aag aaa gcc ttc aac   48
Arg Pro Ser Ser Ile Lys Thr Phe Glu Glu Tyr Lys Lys Ala Phe Asn
 1               5                  10                  15

aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc   96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80

acc aac gcc tgc agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95

cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125

gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140

tgc gcc tcc caa cac gga cgt cat ggg gat acg att ccc aga ggt atc   480
Cys Ala Ser Gln His Gly Arg His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160

gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175

gta gct agg gag cag tcc tgc cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190

att tcc aat tat tgc cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
        195                 200                 205

gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
```

```
aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300

taa                                                               909
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<210> 11
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<400> 11

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Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175
Val Ala Arg Glu Gln Ser Arg Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
        195                 200                 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
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                245                 250                 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
    290                 295                 300


<210> 12
<211> 909
<212> DNA
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<220>
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<222> (1)...(906)

<223> C103R mutant of ProDerP1

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 1               5                  10                  15

aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc   96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80

acc aac gcc tgc agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95

cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125

gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140

tgc gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc   480
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160

gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
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Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
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gta gct agg gag cag tcc cgt cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Arg Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190

att tcc aat tat tgc cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Cys Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
        195                 200                 205

gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
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    210                 215                 220

aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
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taa                                                               909

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<213> Artificial Sequence

<220>
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Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
            100                 105                 110
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
        115                 120                 125
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Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
    130                 135                 140
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
                165                 170                 175
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
            180                 185                 190
Ile Ser Asn Tyr Arg Gln Ile Tyr Pro Pro Asn Val Asn Lys Ile Arg
        195                 200                 205
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
    210                 215                 220
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
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<210> 14
<211> 909
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<223> C117R mutant of ProDerP1

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aag agc tat gcc acc ttc gag gac gag gag gcc gcg cgc aag aac ttc   96
Lys Ser Tyr Ala Thr Phe Glu Asp Glu Glu Ala Ala Arg Lys Asn Phe
            20                  25                  30

ctg gaa agc gtg aaa tac gtg cag agc aac ggc ggg gct ata aat cac   144
Leu Glu Ser Val Lys Tyr Val Gln Ser Asn Gly Gly Ala Ile Asn His
        35                  40                  45

ctg tcc gac ctg tct tta gac gag ttc aag aac cgg ttc ctg atg agc   192
Leu Ser Asp Leu Ser Leu Asp Glu Phe Lys Asn Arg Phe Leu Met Ser
    50                  55                  60

gcc gag gct ttc gaa cac ctt aag acc cag ttt gat ctc aac gcg gag   240
Ala Glu Ala Phe Glu His Leu Lys Thr Gln Phe Asp Leu Asn Ala Glu
65                  70                  75                  80

acc aac gcc tgc agt atc aac ggc aat gcc ccc gct gag att gat ctg   288
Thr Asn Ala Cys Ser Ile Asn Gly Asn Ala Pro Ala Glu Ile Asp Leu
                85                  90                  95

cgc cag atg agg acc gtg act ccc atc cgc atg caa ggc ggc tgc ggg   336
Arg Gln Met Arg Thr Val Thr Pro Ile Arg Met Gln Gly Gly Cys Gly
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            100                 105                 110

tct tgt tgg gcc ttt tca ggc gtg gcc gcg aca gag tcg gca tac ctc   384
Ser Cys Trp Ala Phe Ser Gly Val Ala Ala Thr Glu Ser Ala Tyr Leu
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gcg tat cgg aat cag agc ctg gac ctc gct gag cag gag ctc gtt gac   432
Ala Tyr Arg Asn Gln Ser Leu Asp Leu Ala Glu Gln Glu Leu Val Asp
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tgc gcc tcc caa cac gga tgt cat ggg gat acg att ccc aga ggt atc   480
Cys Ala Ser Gln His Gly Cys His Gly Asp Thr Ile Pro Arg Gly Ile
145                 150                 155                 160

gaa tac atc cag cat aat ggc gtc gtg cag gaa agc tat tac cga tac   528
Glu Tyr Ile Gln His Asn Gly Val Val Gln Glu Ser Tyr Tyr Arg Tyr
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gta gct agg gag cag tcc tgc cgc cgt cct aac gca cag cgc ttc ggc   576
Val Ala Arg Glu Gln Ser Cys Arg Arg Pro Asn Ala Gln Arg Phe Gly
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att tcc aat tat cgt cag atc tac ccc cct aat gcc aac aag atc agg   624
Ile Ser Asn Tyr Arg Gln Ile Tyr Pro Pro Asn Ala Asn Lys Ile Arg
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gag gcc ctg gcg cag acg cac agc gcc atc gct gtc atc atc gga atc   672
Glu Ala Leu Ala Gln Thr His Ser Ala Ile Ala Val Ile Ile Gly Ile
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aag gat ctg gac gca ttc cgg cac tat gac ggg cgc aca atc atc cag   720
Lys Asp Leu Asp Ala Phe Arg His Tyr Asp Gly Arg Thr Ile Ile Gln
225                 230                 235                 240

cgc gac aac gga tat cag cca aac tac cac gcg gtc aac atc gtg ggt   768
Arg Asp Asn Gly Tyr Gln Pro Asn Tyr His Ala Val Asn Ile Val Gly
                245                 250                 255

tac tcg aac gcc cag ggg gtg gac tac tgg atc gtg aga aac agt tgg   816
Tyr Ser Asn Ala Gln Gly Val Asp Tyr Trp Ile Val Arg Asn Ser Trp
            260                 265                 270

gac act aac tgg ggc gac aac ggc tac ggc tac ttc gcc gcc aac atc   864
Asp Thr Asn Trp Gly Asp Asn Gly Tyr Gly Tyr Phe Ala Ala Asn Ile
        275                 280                 285

gac ctg atg atg atc gag gag tac ccg tac gtg gtg atc ctg           906
Asp Leu Met Met Ile Glu Glu Tyr Pro Tyr Val Val Ile Leu
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ctgagattga tctgcgccag atgaggaccg tgactcccat ccgcatgc 108

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<213> Artificial Sequence

<220>
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<400> 16
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<211> 92
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<210> 18
<211> 99
<212> DNA
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<400> 18
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cctgaaaagg cccaacaaga cccacggccg ccttgcatg 99

<210> 19
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<400> 21
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<210> 22
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<220>
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<400> 24
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<210> 25
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<400> 25
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<210> 26
<211> 29
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<213> Artificial Sequence

<220>
<223> Primer

<400> 26
ggtccaggct ctgttgccga tacgcgagg 29

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/09122

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
SEQUENCE SEARCH, EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 25823 A (SMITHKLINE BEECHAM BIOLOG ;BOLLEN ALEX (BE); JACOBS PAUL (BE); BRU) 27 May 1999 (1999-05-27) cited in the application page 4, line 4 - line 13 page 6, line 30 -page 7, line 22; example 2 ---	1-16
X	WO 01 29078 A (BEST ELAINE A ;HESKA CORP (US); MCDERMOTT MARTIN J (US)) 26 April 2001 (2001-04-26) See SEQ ID NO:14 page 63, line 25 - line 29 ---	1-16
A	US 5 670 356 A (SHERF BRUCE A ET AL) 23 September 1997 (1997-09-23) abstract column 9, line 19 -column 10, line 7 --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2002	27/12/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sprinks, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/09122

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 03870 A (HOULBROOK KENNETH) 15 February 1996 (1996-02-15) the whole document	1-16

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/09122

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/09122

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9925823	A	27-05-1999	CA	2309885 A1	27-05-1999
			WO	9925823 A2	27-05-1999
			EP	1032669 A2	06-09-2000
			JP	2001523452 T	27-11-2001
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US 5670356	A	23-09-1997	NONE		
WO 9603870	A	15-02-1996	AT	199476 T	15-03-2001
			AU	687212 B2	19-02-1998
			AU	2749095 A	04-03-1996
			CA	2195978 A1	15-02-1996
			DE	69520275 D1	12-04-2001
			DE	69520275 T2	31-10-2001
			DK	773717 T3	02-07-2001
			EP	1043032 A2	11-10-2000
			EP	0773717 A1	21-05-1997
			ES	2156942 T3	01-08-2001
			WO	9603870 A1	15-02-1996
			PT	773717 T	30-08-2001

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